**The Research Foundation**
of State University of New York

State University of New York at Stony Brook



Susan J. Braman
Jaeckle Fleischmann & Mugel, LLP
Ellwanger and Barry Building
39 State Street
Rochester, NY 14614

**Re: "A genetic screen for protein nuclear import"**
**Inventors: Citovsky, V.; Rhee, Y**
**Our File No: R-7314**

Dear Susan:

I have enclosed a New Technology Disclosure originating from Dr. Vitaly Citovsky's laboratory. He has developed a simple assay to determine whether proteins localize to the nucleus. Because it is less ambiguous and more cost effective, this assay represents an improvement over current methods used to detect nuclear localization of a protein. Please review for obviousness and patentability. I would appreciate your placing a priority on completing this review, as Dr. Citovsky would like to present his work at a meeting on November 7 of this year.

Feel free to contact Dr. Citovsky for any material and literature requests that would assist you in reaching an opinion about the probability of a successful filing. Please copy me on all correspondence and use our NTD titles, reference numbers and inventor's names as referenced above.

1362 (direct)

My telephone number is 516-632-4163. I look forward to hearing from you.

Sincerely,

Margaret Burns

**Margaret Burns, Ph.D.**
Licensing Associate
*Office of technology Licensing & Industry Relations*

## 1. Construction of vectors for the genetic assay:

These constructs are designed to express fusion proteins composed of three functional parts: a modified LexA protein, activation domain of the GAL4 protein, and a protein to be tested for its nuclear import. Below, we describe how these components were obtained and joined together.

(**A**) First, the Gal4 activation domain (AD), derived from the pGAD424 plasmid, was PCR-amplified with and without the adjacent SV40 NLS (AD with NLS was used for positive control constructs, see below). During amplification, EcoRI and BamHI restriction sites were introduced at the 5' and 3' ends of the amplified fragment, respectively. The PCR mixtures contained the following components:

(a) PCR of GAL4 AD without NLS

| | | |
|---|---|---|
| Primer GAD5 | (20uM) | 5 µl |
| PrimerGAD3BdE | (20µM) | 5 µl |
| dNTPs (10mM each for dATP, dTTP, dGTP, dCTP) | | 2 µl |
| Pfu reaction buffer | (10x) | 10 µl |
| Template DNA (pGAD424, 10ng/µl) | | 5 µl |
| Pfu polymerase (0.5 u/µl) | | 1 µl |
| Double distilled water | | 72 µl |
| Total | | 100 µl |

Primer GAD5: 5'-GGGAA TTCAA TTTTA ATCAA AGTGG G-3'
Primer GAD3BdE: 5'-GACGG ATCCC CGGGT ATTCG ATCTC TT-3'

(b) PCR of GAL4 AD with NLS

| | | |
|---|---|---|
| Primer GAD5NLS | (20µM) | 5 µl |
| PrimerGAD3BdE | (20µM) | 5 µl |
| dNTPs (10mM each for dATP, dTTP, dGTP, dCTP) | | 2 µl |
| Pfu reaction buffer | (10x) | 10 µl |
| Template DNA (pGAD424, 10ng/µl) | | 5 µl |
| Pfu polymerase (0.5 u/µl) | | 1 µl |
| Double distilled water | | 72 µl |
| Total | | 100 µl |

Primer GAD5NLS: 5'-GGGAA TTCGA TAAAG CGGAA TTAAT TCCC-3'
Primer GAD3BdE: 5'-GACGG ATCCC CGGGT ATTCG ATCTC TT-3'

PCR conditions for all reactions:

| | |
|---|---|
| 94°C / 2 min. | 1 cycle |
| 94°C / 45sec; 45°C / 45sec; 72°C / 2 min. | 35 cycles |
| 72°C/10 min. | 1 cycle |

(**B**) Then, wild-type LexA in the pBTM116 vector was joined in-frame with Gal4 AD following restriction digestion of the corresponding purified PCR fragments with EcoRI and BamHI using standard molecular biology protocols. The resulting fusion constructs were designated pLexA::GAL4AD (-)NLS and pLexA::GAL4AD (+)NLS.

(C) Next, two testing genes were introduced into pLexA::GAL4AD (-)NLS and pLexA::GAL4AD (+)NLS vectors for NLS-negative and NLS-positive controls.

(a) NLS-negative protein, VirE2 of *Agrobacterium tumefaciens*. VirE2 is known to remain cytoplasmic when expressed in yeast and animal cells, making it a suitable negative control for the nuclear import assay. BamHI fragment of pEE2 plasmid containing the VirE2 ORF was subcloned in-frame into the BamHI site of pLexA::GAL4AD (-)NLS and pLexA::GAL4AD (+)NLS vectors, placing it immediately downstream of GAL4 AD.

(b) NLS-positive protein, VirD2 of *Agrobacterium tumefaciens*. VirD2 is known to accumulate in the cell nucleus when expressed in yeast and animal cells, making it a suitable positive control for the nuclear import assay. BamHI fragment of pED2 plasmid containing the VirD2 ORF was subcloned in-frame into the BamHI site of pLexA::GAL4AD (-)NLS vector, placing it immediately downstream of Gal4 AD.

(D) Finally, the *LexA* gene in the above described constructs was modified to remove its part that encodes a functional nuclear localization sequence (NLS) which had been identified by amino acid sequence analysis of LexA in our lab. This was performed by site directed mutagenesis using Transformer™ Site-Directed Mutagenesis Kit (Cat.# K1600-1) from CLONTECH Laboratories, Inc. according to the manufacturer's protocol. Specifically, two amino acids in the LexA protein were mutated to produce substitutions R157G and K159E by changing their codons CTG to GGC and AAA to GAA, respectively. The sequences for the mutagenesis primers were:

Mutant primer [designated LexA(-NLS)[:

5'-CCGTT AAGGG CCTGG AAAAA CAGGG-3'

Selection primer (designated trans ScaI/StuI):

5'-GTGAC TGGTG AGGCC TCAAC CAAGT C-3'

This procedure produced a modified LexA which was designated mLexA.

Collectively, the described above procedures yielded the following five constructs:

| | |
|---|---|
| 1. pmLexA:: GAL4AD (-)NLS | assay vector, the experimental construct in which the gene of interest should be subcloned in-frame |
| 2. pmLexA:: GAL4AD (-)NLS::VirE2 | negative import control for the assay |
| 3. pmLexA:: GAL4AD (-)NLS::VirD2 | positive import control for the assay |
| 4. pmLexA:: GAL4AD (+)NLS::VirE2 | positive control for the ability of the experimental construct to produce fusion protein capable of nuclear import, i.e. that the protein of interest does not non-specifically alter the conformation of the fusion protein, preventing nuclear import even in the presence of an active NLS. |
| 5. pmLexA:: GAL4AD (-)NLS::2DriV | another negative control containing antisense orientation of VirD2 |

All these plasmids are Amp$^r$ and TRP1, requiring growth on an ampicillin-containing medium in *E. coli* and on a tryptophan drop-out medium in yeast cells.

**2. One-hybrid genetic assay for protein nuclear import using the described above vectors**

(A) It has to be emphasized that:

(a) The fusion protein derived from pLexA::GAL4AD (-)NLS::VirE2 enters the cell nucleus and activates the reporter gene expression, indicating that LexA carries a cryptic NLS although it is a prokaryotic

protein and is not expected to function in the nucleus. Thus it was necessary to identify and disable this signal, resulting in a proprietory modification of the LexA protein (see item D above).

(b) The fusion protein derived from pmLexA:: GAL4AD (-)NLS, which lacks a tested protein, enters the cell nucleus by diffusion due to its small size (approximately 38 kDa). This import, however, is less efficient than that of NLS-containing fusion products, resulting in a weaker expression of the ß-galactosidase reporter. Nevertheless, we suggest using pmLexA:: GAL4AD (-)NLS::VirE2 as a negative control for the assay. This 106 kDa fusion protein does not enter the nucleus at all, producing zero expression of the reporter.

(c) The user has an option of constructing his/her own custom made negative control for this assay by subcloning the protein of interest in antisense orientation. Our results indicate that VirD2, which targets the fusion product derived from pmLexA:: GAL4AD (-)NLS::VirD2 to the nucleus, does not promote nuclear import when subcloned into the same vector in antisense orientation, i.e. pmLexA:: GAL4AD (-)NLS::2DriV.

(d) The current version of pmLexA:: GAL4AD (-)NLS includes only three unique cloning sites, BamHI, SalI, and PstI, for insertion of the gene of interest. However, additional sites can be easily engineered, if required, using simple standard cloning techniques.

**(B)** Once the gene of interest is inserted in-frame into the pmLexA:: GAL4AD (-)NLS assay vector, it can be transformed into the L4O yeast strain (MATα his3Δ200 trp1-90 leu2-3,112 ade2, lys2::LYS2::lexAHIS3, ura3::URA3::lexA lacZ gal80) by any standard procedure using either Lithium acetate or electroporation (Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, and K. Struhl, *Current Protocols in Molecular Biology*. 1987, New York: Greene Publishing-Wiley Interscience). For negative and positive controls, the appropriate constructs (described above) are separately introduced into L4O cells.

**(C)** The resulting yeast strains are grown on a selective medium and assayed for ß-galactosidase activity after one or two days of growth using standard procedures. Positive result, i.e. dark blue-stained yeast colonies, indicates active nuclear import of the fusion protein and, consequently, the presence of a functional NLS in the tested protein.

### 3. Specific questions about characteristics of the key elements of the constructs

**(A)** *Can the ADH1 promoter be replaced with another promoter? What characteristics describe the suitable promoter?*

The main feature of the promoter is the strength of expression. ADH1 is a strong constitutive promoter but inducible promoter such as GAL promoter may be used.

**(B)** *Can the LexA or GAL4 genes be replaced with some other gene? What characteristics describe the function of the LexA or GAL4 gene, and could generally describe that element of the vector?*

There are two main principles to be protected in this patent: The first principle is to have a DNA binding domain (recognizing a specific promoter region) and a gene activation domain (activating that same promoter) in its final fusion protein. In the final configuration, i.e. when fused to the tested protein, these domains should be insufficient for the entire fusion product to enter the yeast cell nucleus and activate gene expression. In our construct, the binding domain is supplied by LexA and the activation domain is derived from GAL4. In principle, however, the source of these domains can be modified (e.g. from a well characterized transcription factor, although we do not know of one more or equally suitable than the ones chosen for our construct). Importantly, the use of any other promoter binding and activating proteins must ensure that the do not contain

functional NLS sequences (note that most transcription factors do possess NLSs since they function in the cell nucleus).

The second principle is our proprietary modification of LexA which abolishes its intrinsic NLS activity but preserves its ability to bind promoter elements. We made two specific amino acid changes in the LexA primary sequence but, in principle, it may be possible to alter other amino acids in LexA to achieve the same objectives, i.e. block the NLS function but retain the specific DNA binding to the LexA operators of the promoter. Thus, patenting our modification of LexA is essential since it for the first time uncouples nuclear import and promoter binding activities of LexA.

(C) *What are the characteristics of the reporter gene and the yeast strain? Can alternative reporters be used?*

Our assay has two built-in reporters. The first reporter is the ß-galacosidase enzyme. It is induced only after the tested protein-containing fusion product enters the cell nucleus, resulting in strong blue color of the yeast colonies. In addition, nuclear import of the fusion protein induces an auxotrophic marker HIS3, resulting in the ability of the yeast cells to grow on a histidine-deficient medium. Since histidine selection is known to be slightly leaky, the best results are achieved by including 3-amino-1,2,4,-triazole in the growth medium. Generally, we recommend using both markers for the assay.

Our assay utilizes the yeast strain L4O. This strain is the most suitable for the assay because it contains both reporter genes under inducible promoters activated by the LexA-GAL4AD fusion. This strain also is unable to grow in the absence of tryptophan and/or histidine, allowing for selective growth of cells containing the assay plasmid (TRP1 marker) and/or induced reporter gene (HIS3 marker). Potentially, another strain can be engineered to conform to these requirements although we know of none such available strain at present. If the assay cassette, i.e. the fusion gene mLexA::GAL4AD(-)NLS, is transferred to another vector with a different auxotrophic growth marker (e.g., LEU or URA), the host strain has to be modified accordingly to allow the selective growth of the new plasmid.

Whereas we feel that our reporter genes are adequate and even optimal for the assay, they can be substituted with other reporters. For example, ß-galacosidase can be exchanged with green fluorescent protein (GFP) and HIS3 replaced with URA3. Although GFP detection does not require specific staining used for the ß-galacosidase assay, the latter may be more easily and accurately quantified. This quantification may be useful for comparisons of NLS activities between different proteins of interest.

(D) *Plasmid maps*

See attached maps for pBTM116, pGAD424, pEE2, pED2, pmLexA:: GAL4AD (-)NLS, pmLexA:: GAL4AD (-)NLS::VirE2, pmLexA:: GAL4AD (-)NLS::VirD2, pmLexA:: GAL4AD (+)NLS::VirE2, and pmLexA:: GAL4AD (-)NLS::2DriV.

10. BRIEF TECHNICAL CONFIDENTIAL DESCRIPTION

## Protein Nuclear Import Assay

A yeast one-hybrid expression vector, designated pLG, was designed to conveniently and rapidly assay the ability of proteins to enter the cell nucleus. pLG expresses a triple-fusion protein comprising bacterial LexA, yeast Gal4 activation domain, and the tested protein encoded by a cDNA subcloned in-frame into the multiple cloning site downstream of Gal4 activation domain open reading frame (ORF) (Figure 1A).

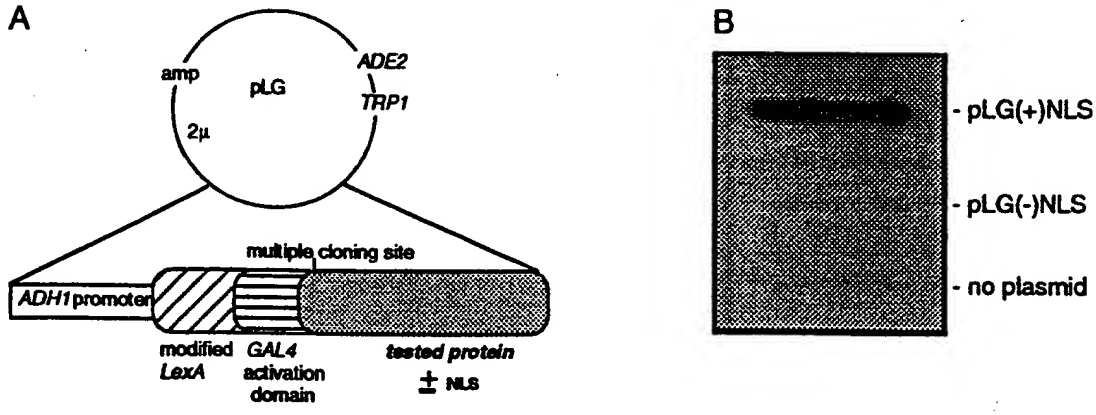



**Figure 1.** One-hybrid protein nuclear import assay. **(A)** pLG plasmid used for the assay. **(B)** ß-galactosidase assay to detect nuclear import of the tested protein.

If the tested protein contains a functional nuclear localization signal (NLS), the fusion protein will enter the yeast cell nucleus. Following this nuclear import, the LexA domain targets the fusion protein to the LexA operator sites of the reporter *lacZ* gene contained in the L40 yeast strain. The Gal4 activation domain then activates the expression of *lacZ*, resulting in ß-galactosidase activity (Figure 1B). In the absence of NLS, the fusion construct is unable to reach the cell nucleus and, thus, activate the reporter gene. Indeed, expression of pLG carrying a cDNA for a non-nuclear protein does not produce any detectable ß-galactosidase activity (Figure 1B).

In addition to induction of the ß-galactosidase reporter, this one-hybrid system allows to directly select for the nuclear import of the tested protein in the same L40 yeast strain, which contains an integrated copy of the *his3* gene with upstream LexA operators. Similarly to the ß-galactosidase expression, only cells expressing the NLS-containing fusion protein are able to grow on a histidine-deficient medium (data not shown).

The key component of the pLG vector is a modified LexA gene which was produced in our laboratory. Clearly, the success of this nuclear import assay hinges on the inability of LexA::Gal4::tested protein fusions to enter the cell nucleus in the absence of an NLS. Thus, neither LexA nor Gal4 should contain NLS sequences. Indeed, the Gal4 activation domain is known to lack NLS whereas LexA, a bacterial protein, is generally thought not to have evolved such signal. However, our studies demonstrated that wild-type LexA carries a previously unidentified NLS sequence, rendering the above described experimental design impossible. To circumvent this difficulty, we identified the LexA NLS and inactivated it by specific substitutions of two amino acid residues. This proprietary modification of LexA is the critical aspect of the one-hybrid nuclear import assay.

We suggest that pLG and L40 can be offered as a kit for simple and rapid functional assay of nuclear import. In addition, this kit should contain a positive control for nuclear import. Thus, we constructed a pLG derivative containing the SV40 NLS sequence at the LexA:Gal4 junction. Fusion proteins produced from this construct always localize to the nucleus, resulting in *lacZ* expression and cell growth in the absence of histidine. This control construct, therefore, is designed to demonstrate the functionality of the assay as well as the active conformation of the fusion protein.

## 14. NON-CONFIDENTIAL DESCRIPTION OF THE TECHNOLOGY

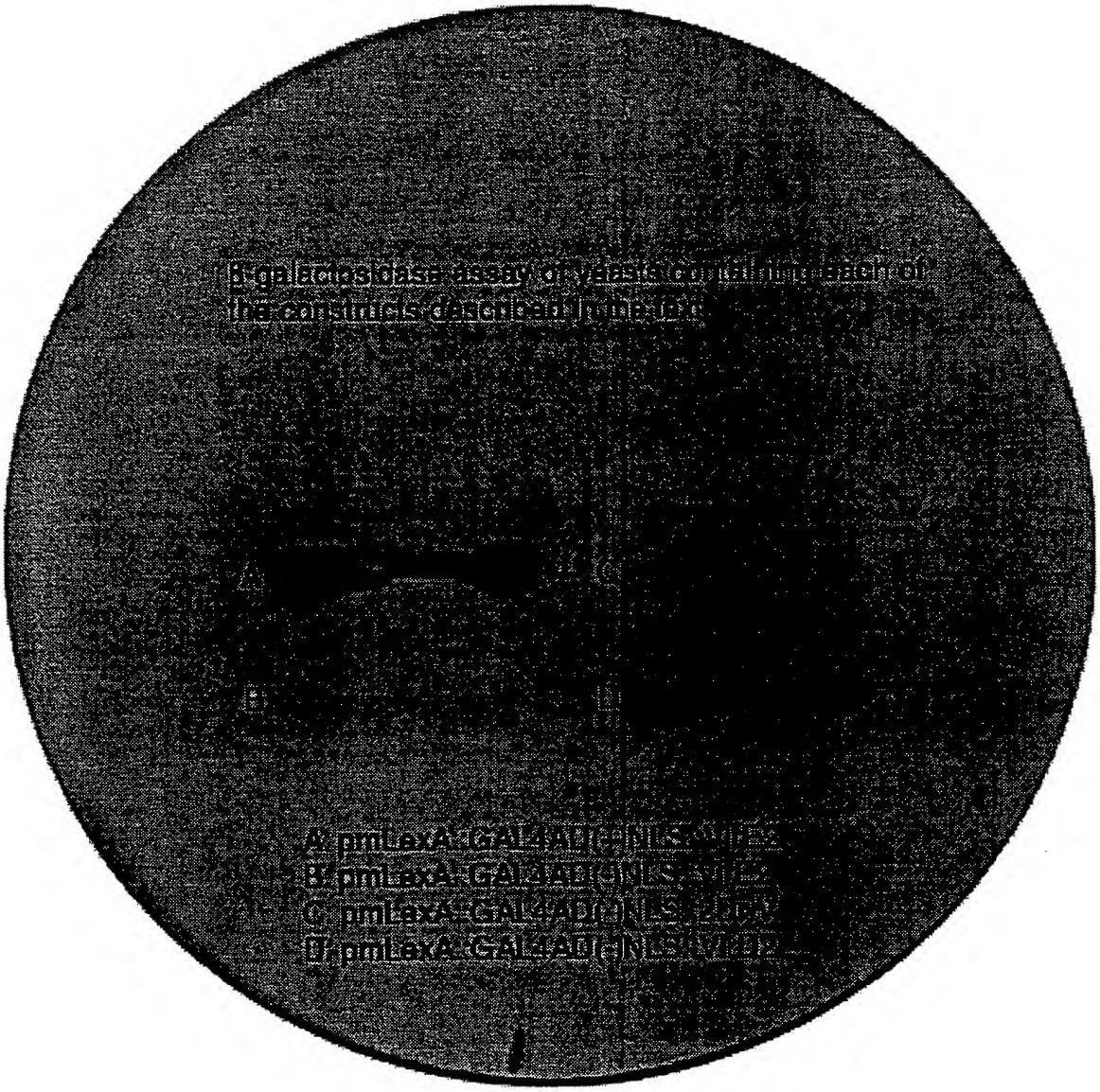
### Protein Nuclear Import Assay

Nuclear import of protein molecules is a basic biological process central to regulation of gene expression which underlies all aspects of development, morphogenesis, and signaling pathways in eukaryotic organisms. Furthermore, nuclear import is an essential step in many host-pathogen interactions such as viral and bacterial infection. It is natural, therefore, that many researchers are interested whether their newly-cloned genes may encode a protein that functions within the cell nucleus.

Two major experimental approaches have been developed to address this question. First, the protein of interest is labeled, microinjected into eukaryotic cells, and its intracellular localization determined. Alternatively, the tested genes are fused to a reporter (ß-galactosidase, green fluorescent protein, etc.), expressed in eukaryotic cells, and the localization of the resulting fusion protein determined. Both methods have serious technical disadvantages. The first approach is very labor-intensive and requires highly trained personnel experienced in protein purification, microinjection, and fluorescent or electron microscopy techniques. The second method is also very laborious, involving often elaborated procedures for genetic transformation of higher eukaryotic cells and microscopy observations.

Recently, we have devised a simple functional assay for protein nuclear import which circumvents all of the above mentioned difficulties. Briefly, we use a specifically modified yeast one-hybrid expression plasmid into which a gene of interest can be easily subcloned to produce a fusion protein in an appropriate yeast strain. If the tested gene product is a nuclear protein, its expression in yeast cells will enable them to grow on a nutrient-deficient medium as well as produce blue colonies following a simple ß-galactosidase staining protocol.

Our one-hybrid system can be packaged as a protein nuclear import assay kit consisting of our proprietary expression vector, a suitable yeast strain, and a positive control construct. Because protein nuclear transport is conserved in fungal, plant, and animal cells, our system will be of use to scientists working in all eukaryotic experimental systems.


β-galactosidase assay of yeasts containing each of the constructs described in the text.
A: pmLexA-GAL4AD(c)NLS-VP16
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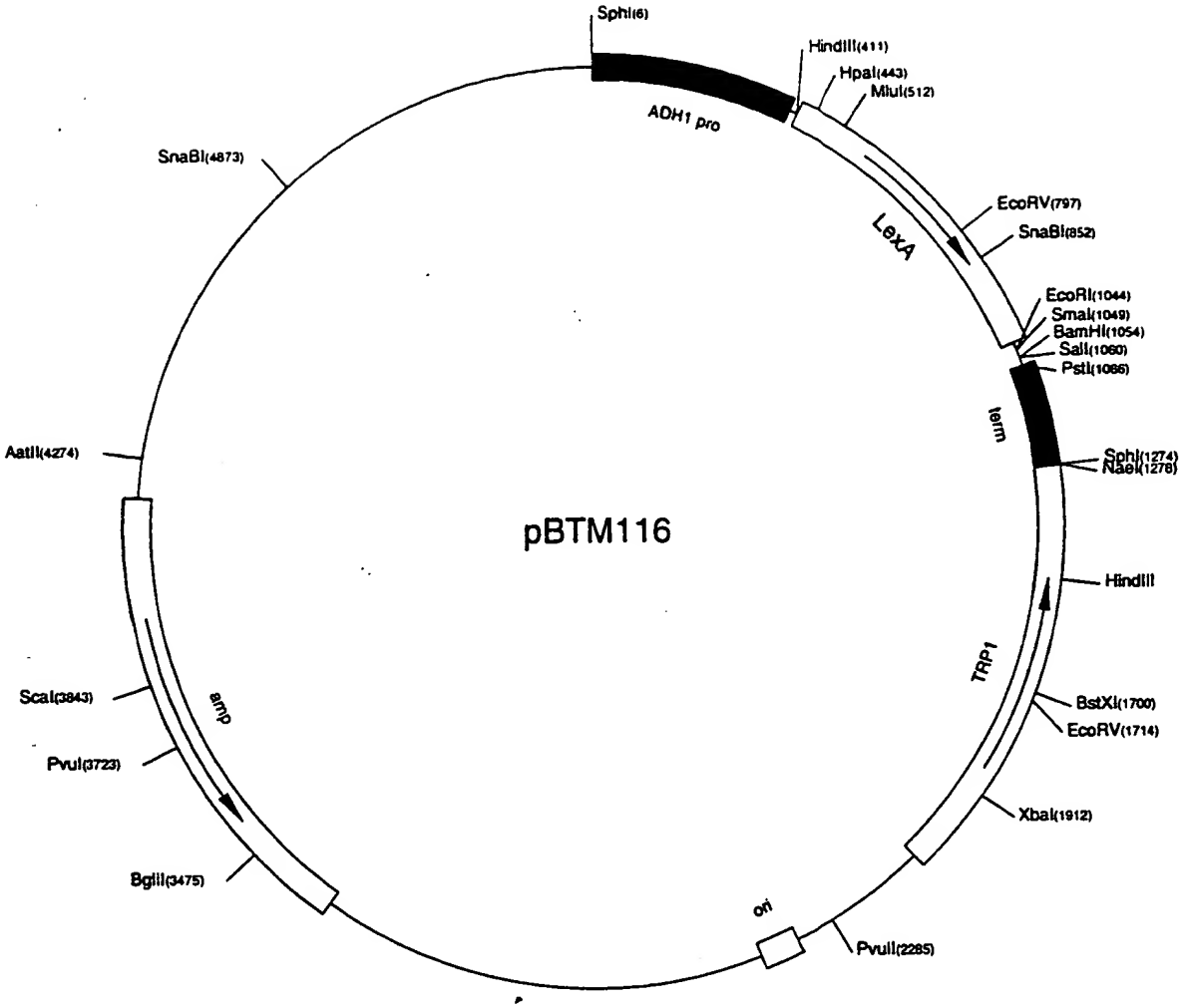
C: pmLexA-GAL4AD(c)NLS-VP16
D: pmLexA-GAL4AD(c)NLS-VP16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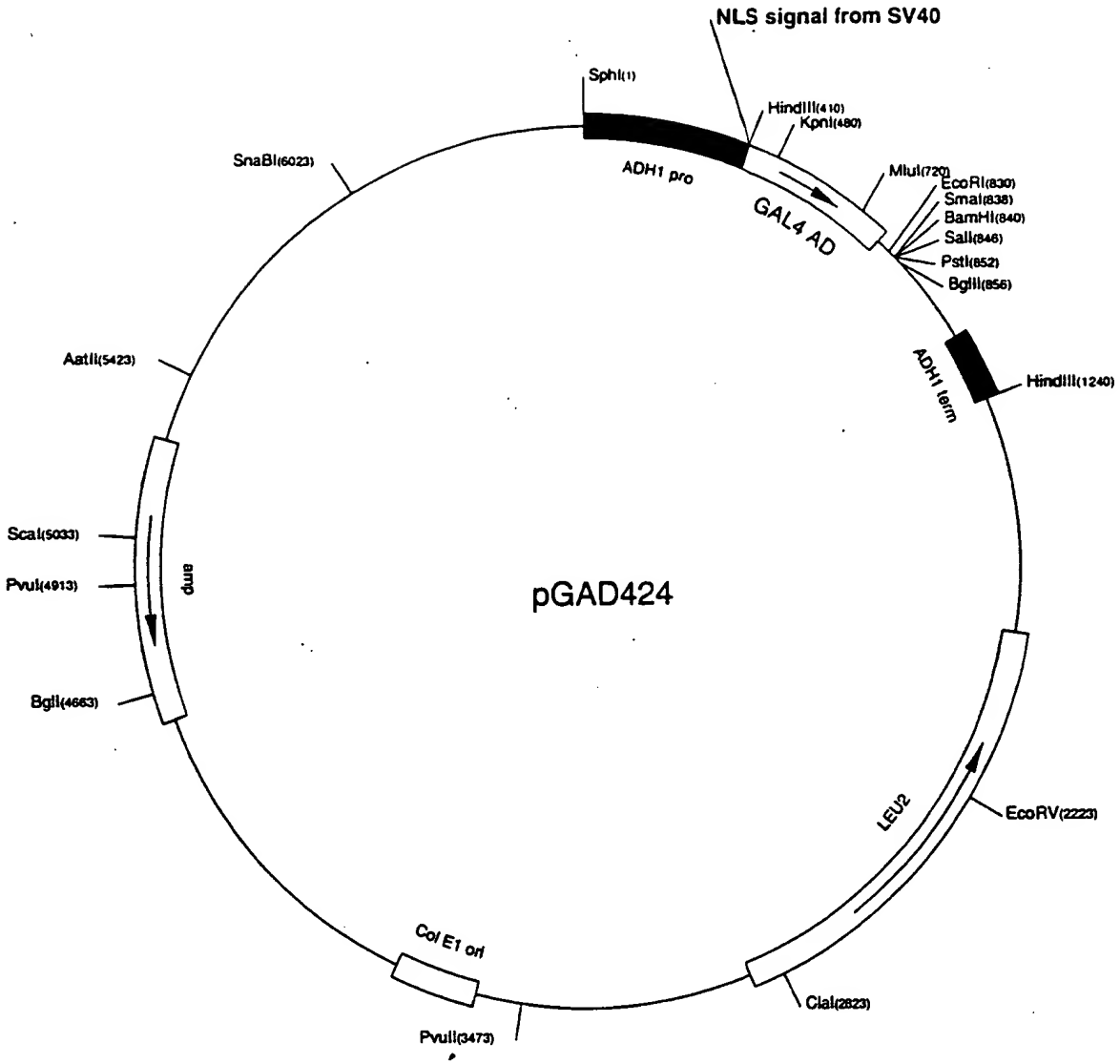
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ClaI(2823)
Col E1 ori
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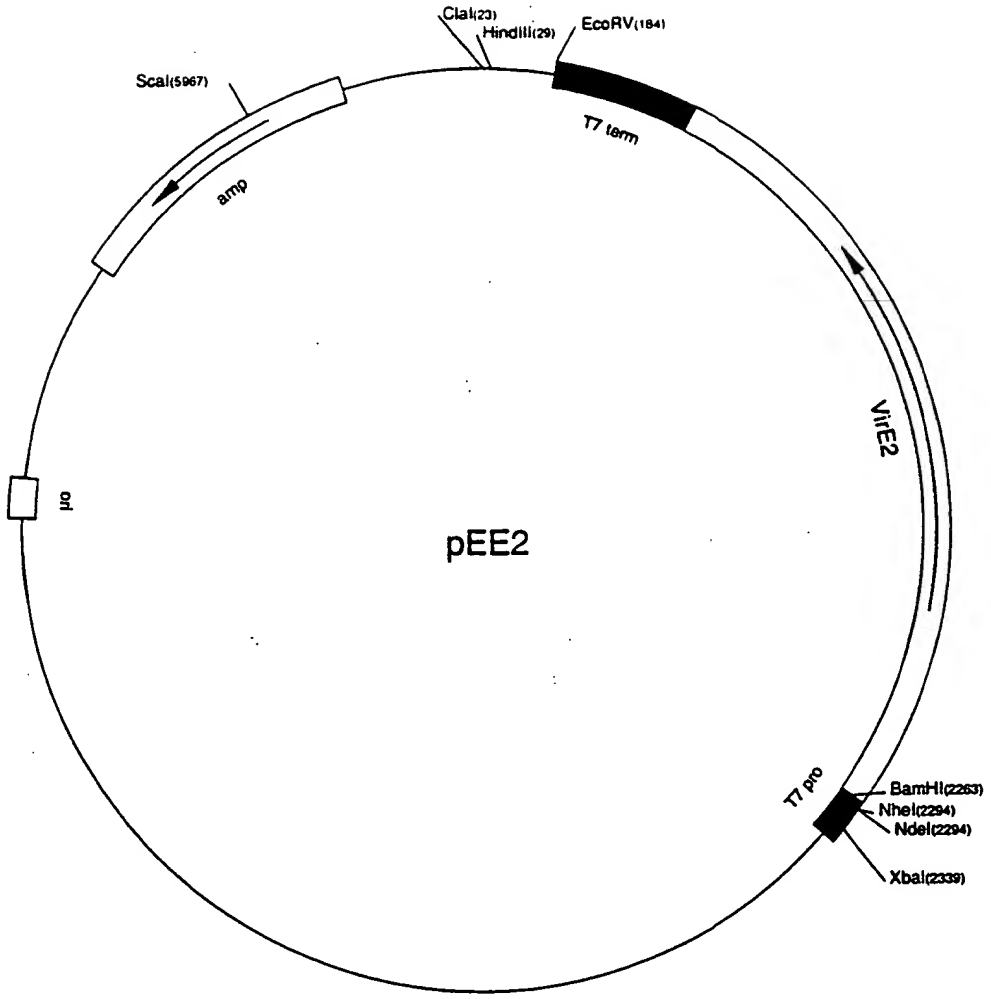
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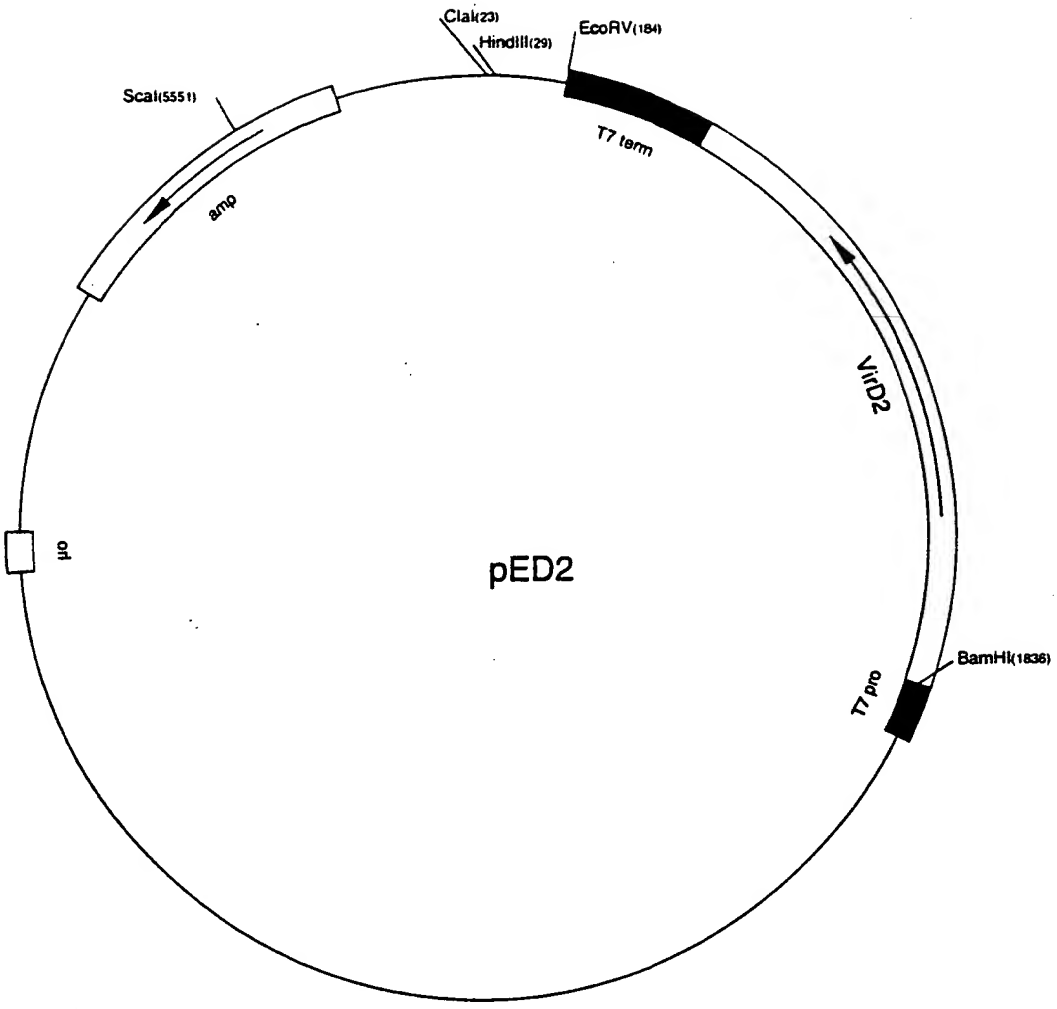
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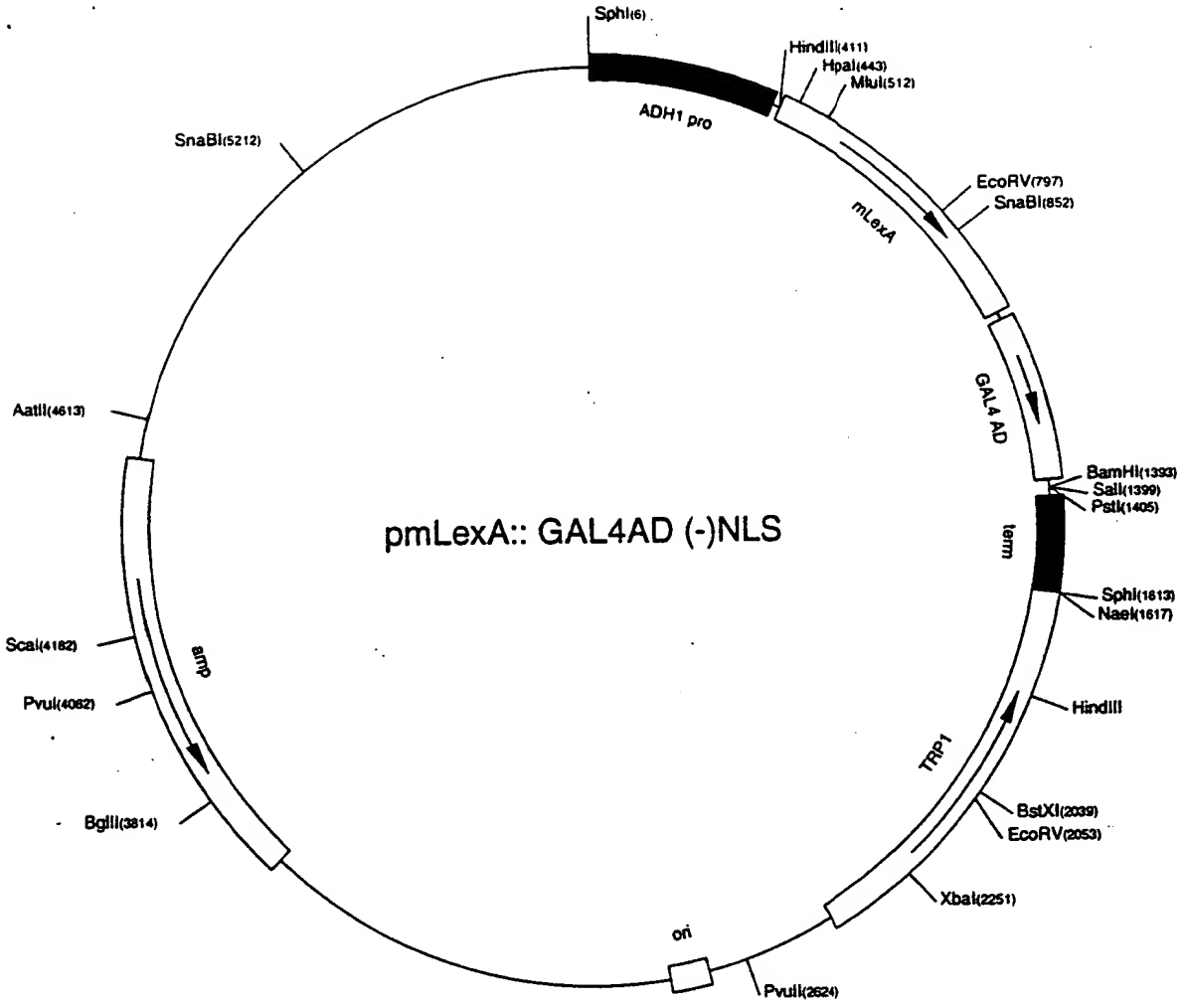
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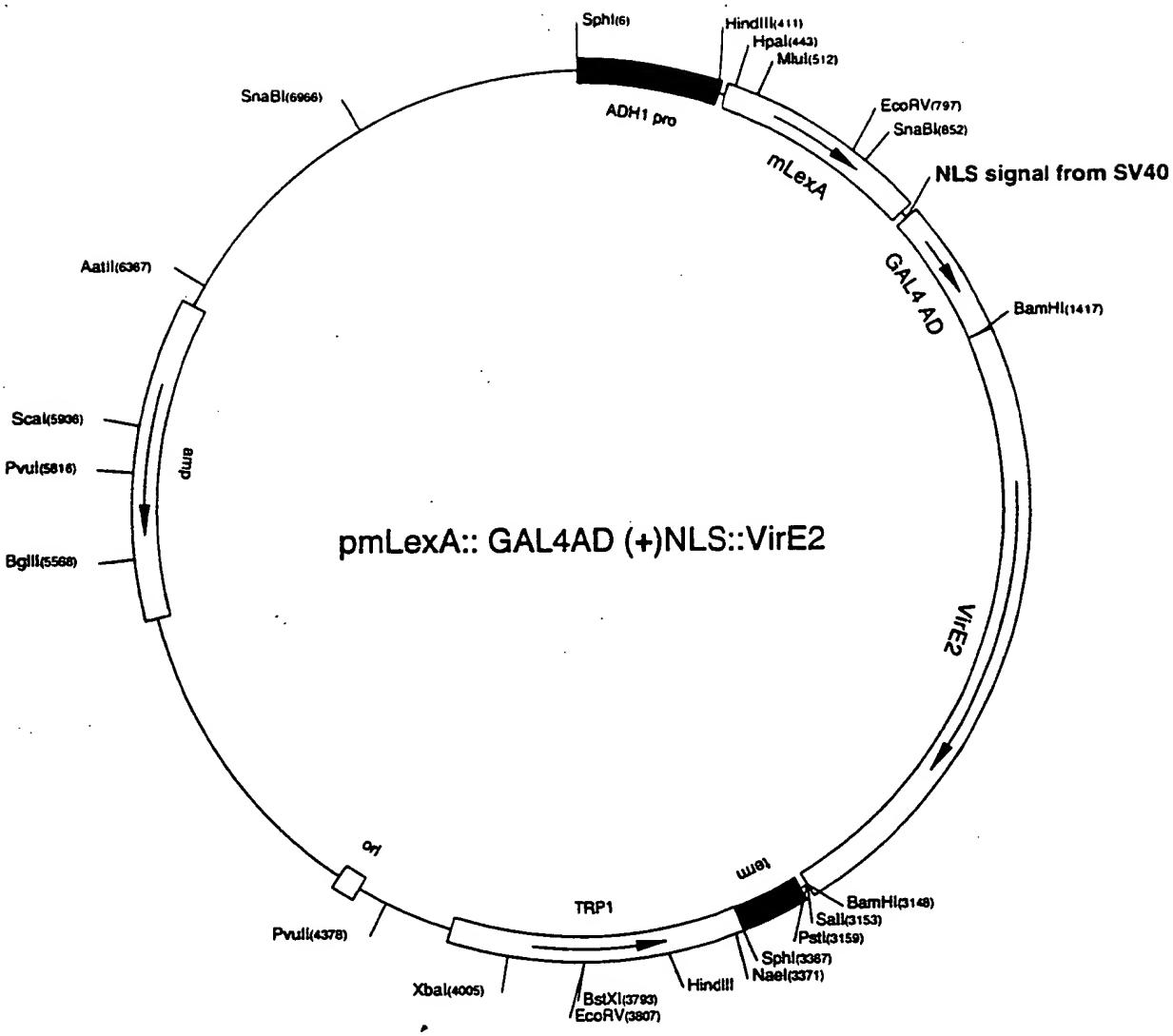
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NLS signal from SV40
BamHI(1417)
SnaBI(6966)
ADH1 pro
mLexA
GAL4 AD
AatII(6367)
ScaI(5936)
PvuI(5816)
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amp
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VirE2
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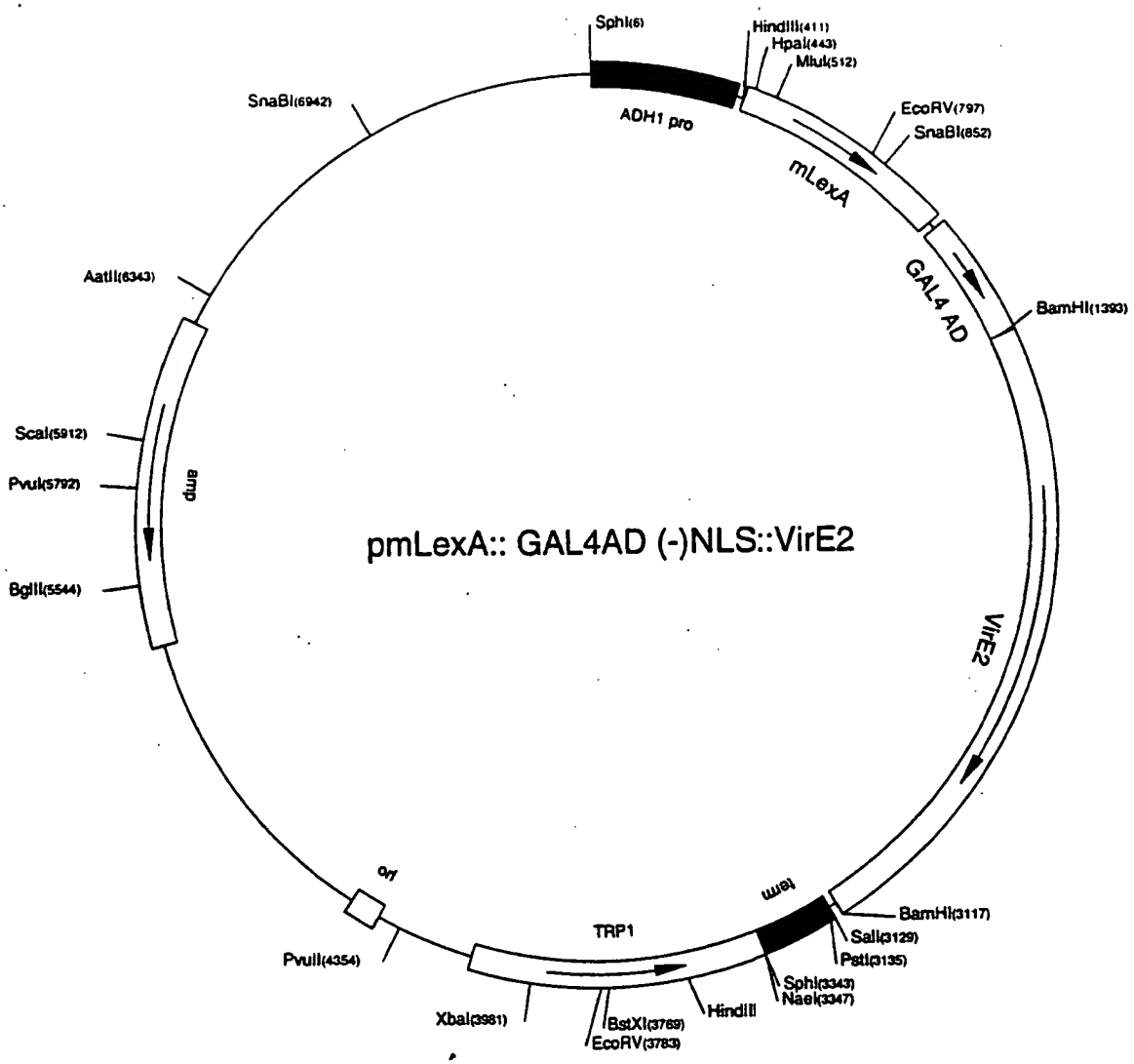
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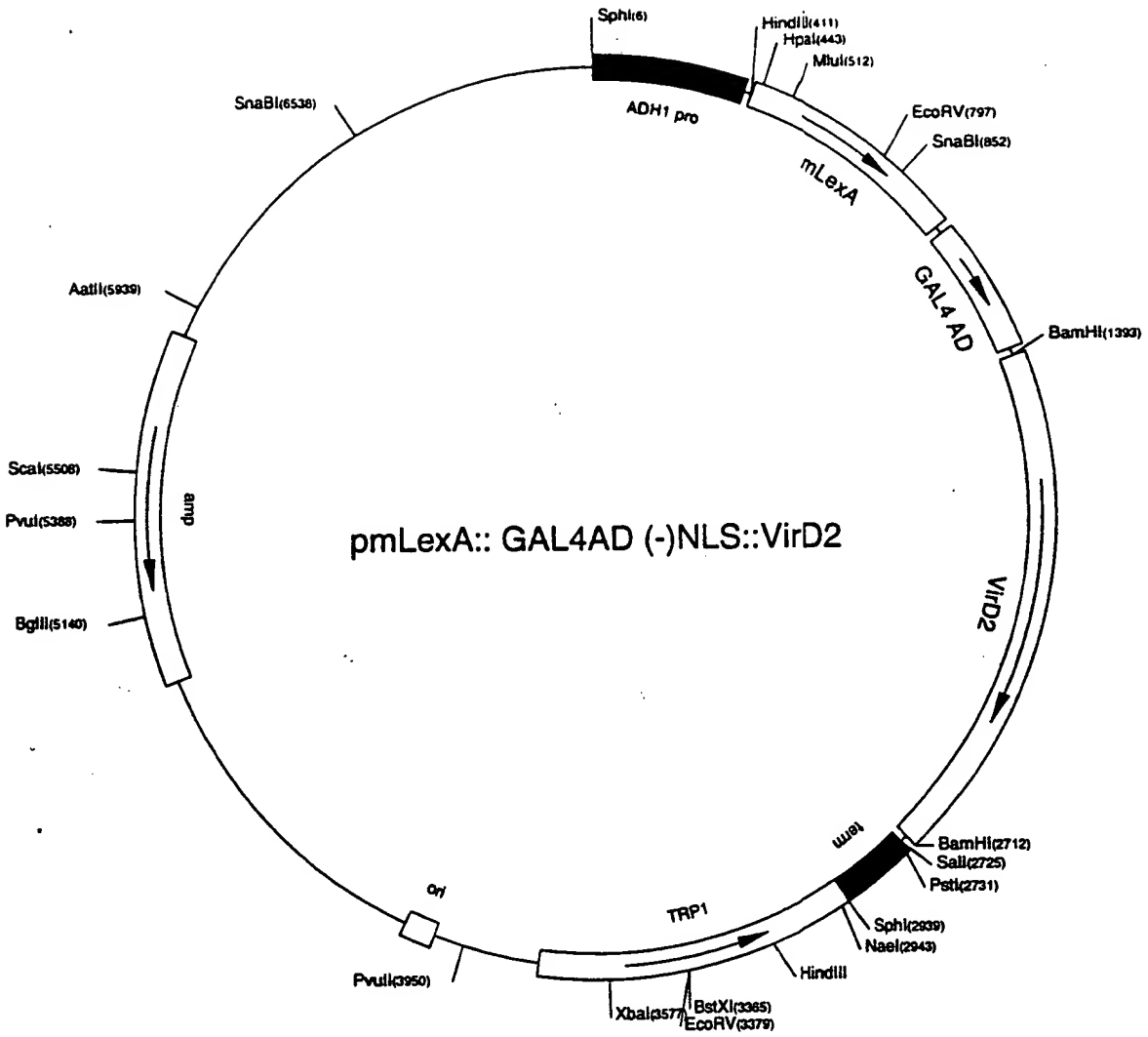
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VirD2
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TRP1
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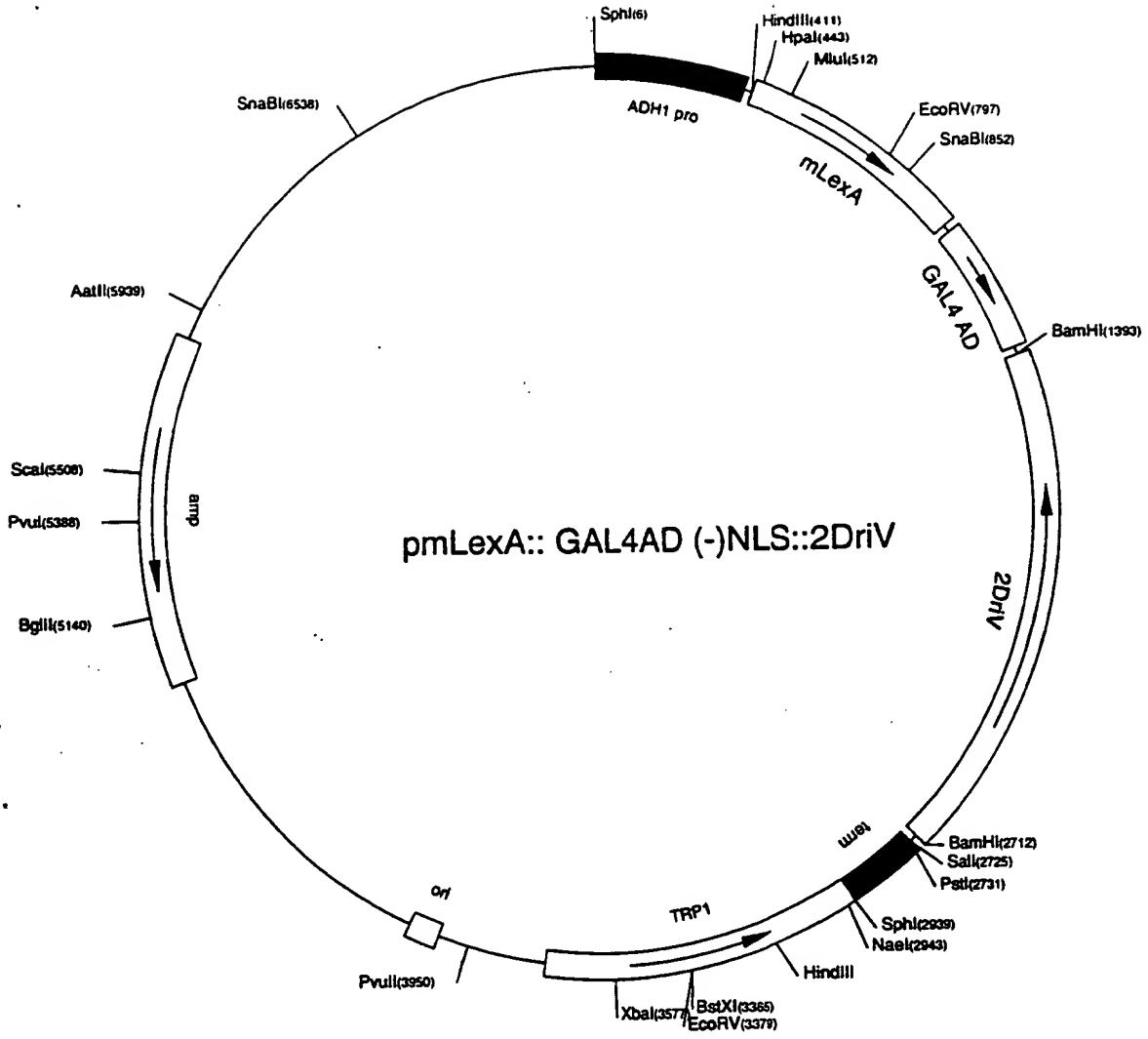
amp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HpaI(443)
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EcoRV(797)
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BamHI(1393)
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PvuI(5388)
BglII(5140)
ADH1 pro
mLexA
GAL4 AD
2DriV
TRP1
ori
amp
ampR